Cell-Based Assays to Detect the Mechanism of Toxicity

Terry Riss, PhD
**Presentation outline**

- Overview of cytotoxicity assays for multi-well plates
  - Live cells
  - Dead cells
  - Apoptotic cells
- Biochemical markers of cell stress
- Multiplexing assays to get more information
- Genetic reporter assays to detect stress response pathways
How do I choose the most appropriate assay?

First decide what you want to measure

- Number of living cells (viability assay)
- Number of dead cells (cytotoxicity assay)
- Apoptosis vs. necrosis
- Determine events leading up to apoptosis
Understanding how the assays work

Understanding:

• what the assay is measuring
• how the reagent chemistry works

Will help you predict:

• assay limitations
• potential for artifacts
• compatibility for multiplexing
Methods for measuring cytotoxicity

Overview of assays to measure viable cells
- MTT / MTS / Resazurin
- Protease marker
- ATP

Assays to detect dead cells
- LDH release
- Protease release
- DNA staining

Assays to measure apoptotic cells
- Caspase activity

Multiplex assays to measure early markers of cytotoxicity
- Viable, dead & apoptotic cells
- Extrinsic & intrinsic apoptosis pathways
- Mitochondrial toxicity
- Oxidative stress
- Proteasome activity

Luciferase reporters of cell stress pathways leading to cytotoxicity
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Luciferase reporters of cell stress pathways leading to cytotoxicity
**Common metabolic indicators of cell viability**

- **Indicator dyes (MTT / MTS / Resazurin):** NADH in metabolically active viable cells can reduce tetrazolium compounds into brightly colored formazan products or reduce resazurin into fluorescent resorufin.

- **Protease marker:** Aminopeptidase activity present only in viable cells can be measured using a cell permeable fluorogenic substrate.

- **ATP:** is present in all cells and has been established as a valid marker of cell viability. ATP is measured using a beetle luciferase reaction to generate light.
Metabolic indicators of cell viability

Tetrazolium Reagent

Tetrazolium Reagents
- MTT
- MTS
- XTT
- WST-1

Viable Cell
- Active Metabolism
- NADH → NAD⁺

Dead Cell
- Marker is absent
- Substrate → No Rxn

Tetrazolium Reagent
- Formazan

Promega
Metabolic indicators of cell viability

Redox Indicators
- Resazurin

Viable Cell
- Active Metabolism
- NADH, NAD⁺
- Resazurin → Resorufin

Dead Cell
- Marker is absent
- Substrate
- No Rxn

Resazurin
Prolonged exposure of cells to tetrazolium or resazurin reagents results in toxicity

Viability (ATP Content) of HepG2 Cells After Exposure to Resazurin

Toxicity detected at 4 hours incubation
Metabolic indicators of cell viability

GF-AFC

- Enzyme Substrates
  - Protease Substrates

Viable Cell
- Active Metabolism
  - aminopeptidase

GF-AFC

AFC

Dead Cell
- Marker is absent
- Substrate
- No Rxn

GF-AFC
ATP assay for cell viability

CellTiter-Glo® Reagent

- Lysis Solution
- ATPase Inhibitors
- Luciferin
- UltraGlo Luciferase

Viable Cell

ATP

Luciferin + Luciferase

Light

Dead Cell

ADP

No Reaction

X
## Advantages & disadvantages of viability assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>MTT / MTS</td>
<td>Widely used</td>
<td>1-4 hour incubation</td>
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<td></td>
<td>Inexpensive</td>
<td>Interference by reducing compounds</td>
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<tr>
<td></td>
<td></td>
<td>2 step protocol (MTT)</td>
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<td></td>
<td></td>
<td>Toxic to cells</td>
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<tr>
<td></td>
<td></td>
<td>Limited sensitivity</td>
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<tr>
<td>Resazurin</td>
<td>Inexpensive</td>
<td>1-4 hour incubation</td>
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<tr>
<td></td>
<td>Fluorescent readout</td>
<td>Interference by reducing compounds</td>
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<tr>
<td></td>
<td>Good sensitivity</td>
<td>Toxic in some cases*</td>
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<tr>
<td></td>
<td></td>
<td>Fluorescence interference</td>
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<tr>
<td>Protease</td>
<td>30 min protocol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Better sensitivity than resazurin</td>
<td>Fluorescence interference</td>
</tr>
<tr>
<td></td>
<td>Cells remain viable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Good choice for multiplexing</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>10 min protocol</td>
<td>Lytic protocol dictates sequence for multiplexing</td>
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<tr>
<td></td>
<td>Best sensitivity</td>
<td></td>
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<tr>
<td></td>
<td>No interference by fluorescent compounds</td>
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<tr>
<td></td>
<td>Stops reaction immediately</td>
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</tr>
</tbody>
</table>
**Methods for measuring cytotoxicity**

Overview of assays to measure viable cells
- MTT / MTS / Resazurin
- Protease marker
- ATP

**Assays to detect dead cells**
- LDH release
- Protease release
- DNA staining

Assays to measure apoptotic cells
- Caspase activity

**Multiplex assays to measure early markers of cytotoxicity**
- Viable, dead & apoptotic cells
- Extrinsic & intrinsic apoptosis pathways
- Mitochondrial toxicity
- Oxidative stress
- Proteasome activity

**Luciferase reporters of cell stress pathways leading to cytotoxicity**
Assays to detect dead cells

The functional definition of cell viability is based on whether the outer membrane is intact.

Membrane integrity and thus dead cells can be detected by:

- Measuring activity of marker enzymes that leak out of dead cells into the culture medium.
- Observing staining of cytoplasmic or nuclear content by vital dyes that can only enter dead cells.
Loss of membrane integrity defines a “dead” cell

Trypan blue staining to determine percent viability may be the most common “assay” used in cell culture labs.

Trypan blue is not permeable to live cells.

Dead cells that have lost membrane integrity take up dye and stain blue.
LDH release assay to detect dead cells

CytoTox-ONE™ Reagent*

- Lactate
- NAD⁺
- Diaphorase
- Resazurin

Viable Cell

Dead Cell

Enzymes retained in live cells

No Reaction

LDH

Fluorescence

*US Patent 6,982,152 B2
LDH assay chemistry

Lactate $\xrightarrow{LDH} \text{Pyruvate}$

$\text{NAD}^+ \xleftarrow{\text{Diaphorase}} \text{NADH}$

Necrotic cell

LDH

Resorufin $\xrightarrow{\text{Resazurin}}$
Stability of released enzyme activity in culture medium becomes a limitation

LDH-Release Assay Time Course
Tamoxifen Treated HepG2 Cells

Cells are all dead at 2 hr treatment with 150µM Tamox

LDH activity in medium decreases after 24 hours

Protease release assay to detect dead cells

- Impermeable substrate cannot enter viable cells
- "Dead" Cell protease remains active after cell death
- The only signal is from "Dead" Cells

bis-AAF-R110 (no added enzymes)

Viable Cell

Dead Cell

No signal from viable Cells

"Dead Cell" Protease

Active "Dead Cell" Protease

bis-AAF-R110

bis-AAF-R110

Fluorescence
485/520nm
DNA dye staining to detect dead cells

Non-permeable DNA dye

Staining of dead cells results in a fluorescent signal.

Viable Cell
Dye is excluded from live cells

Dead Cell
DNA dye only stains nucleus of “dead” cells or debris
Advantage of DNA staining = signal stability

- DNA staining of dead cells produces a fluorescent signal that lasts much longer than the signal from enzyme release assays.
- When marker enzymes are released from dead cells, enzymatic activity diminishes over time in culture medium (~9hr half-life)
- Promega’s new DNA staining dye overcomes the major disadvantage of enzyme release assays.
- CellTox Green product will be commercially available soon
Promega’s DNA dye is not toxic to cells

Incubation of DNA dye with cells for 72 hours has no effect on viability measured using the CellTiter-Glo ATP Assay.

DNA dye does not effect IC_{50} of model compounds in 72hr co-incubations.
Add DNA dye when seeding cells

- Incubate 72hr
- Record fluorescence from dead cells
- Add CellTiter-Glo® Reagent
- Record Luminescence from live cells
## Advantages & disadvantages of assays to detect dead cells

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<tr>
<td></td>
<td>Absorbance or fluorescent options</td>
<td>Limited half-life of LDH in medium</td>
</tr>
<tr>
<td>Protease release</td>
<td>Designed for multiplexing</td>
<td>Limited half-life of protease marker</td>
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<tr>
<td></td>
<td>More sensitive than LDH</td>
<td>Fluorescence interference (fluorescent format only)</td>
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<tr>
<td></td>
<td>Fluorescent reagent is simpler than formulation for LDH assay</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fluorescent or luminescent options</td>
<td></td>
</tr>
<tr>
<td>DNA Staining</td>
<td>Non-toxic / real time assay</td>
<td>Fluorescence interference</td>
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<tr>
<td></td>
<td>Staining persists for 72 hours</td>
<td>Less sensitive than amplified protease release assay</td>
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<td></td>
<td>Good choice for multiplexing</td>
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Multiplex assays to measure early markers of cytotoxicity

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- Oxidative stress
- Proteasome activity

Luciferase reporters of cell stress pathways leading to cytotoxicity
Assays to detect apoptotic cells

- Apoptosis is a form of programmed cell death that can be triggered by a variety of signal transduction pathways.
- Caspase family members are involved in upstream signal transduction events as well as the systematic dismantling of structural and functional components of the cell.
- Caspase-3 is the predominant “executioner” protease in apoptotic cells and is considered a “universal” marker of apoptosis.
- Caspase-3 activity can be easily measured using homogeneous fluorescent or luminescent multi-well plate assays.
AMC, R110 and aminoluciferin substrates for measuring caspase activity

Caspase 3

Fluorescence

Linuminescence

Luciferase + ATP
**Bioluminescent protease assay chemistry**

Z-Peptide- $\text{N}^\text{H}$

\[
\begin{align*}
\text{Protease} & \quad \rightarrow \\
\text{Z-Peptide-} + & \quad \rightarrow \\
\text{Luciferase} & \quad \rightarrow \text{ATP, Mg}^{++} \quad \text{Oxygen} \\
\end{align*}
\]

Peptide-aminoluciferin is not a substrate for luciferase.
Luminescent caspase assay

Caspase-Glo® Reagent

- Lysis Solution
- Z-DEVD-aminoluciferin
- Stable Luciferase
- ATP

Viable Cell

- Inactive Caspase

Apoptotic Cell

- Active Caspase

Necrotic Cell

- Inactive Caspase

Reagent
- No Rxn

Luminescence
Fluorescent caspase assay for apoptosis

X-DEVD-Fluor
Reagent

- Caspase Substrate
- Lysis Solution

Viable Cell
- Inactive Caspase
- Reagent
- No Rxn

Apoptotic Cell
- Active Caspase
- Reagent
- Fluorescence

Dead Cell
- Inactive Caspase
- Reagent
- No Rxn
Comparison of luminescent and fluorescent protease assay sensitivity

Same “DEVD” protease substrate, but three different “R” groups

Luminescence is 50-1000-fold more sensitive than fluorescent assays
Low background & long linear range of response
Methods for measuring cytotoxicity

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Luciferase reporters of cell stress pathways leading to cytotoxicity
Multiplexing is...

Gathering more than one set of data from the same sample

**Multiplexing requirements:**

- Assays must be biologically & chemically compatible
- Signals must be spectrally distinct
Why do multiplexing?

Multiplexing gives a more complete picture of what’s happening in the sample

• Reduces cell culture effort to do more than one assay on the same sample of cells

• Can confirm results with two independent methods

• Can normalize assay signal to viable (or total) cell number
  • Correct for cell dispensing errors
  • Differential growth of cells & edge effects
Multiplex detection of viable, dead and apoptotic cells to determine mechanism of cell death

**Primary necrosis**
(No caspase activity)

- GF-AFC (Viability) EC$_{50}$ = 6.89µM
- bis-AAF-R110 (Cytotoxicity) EC$_{50}$ = 6.87µM
- Caspase-Glo 3/7 (Apoptosis) EC$_{50}$ = ND

**Apoptosis**
(Secondary necrosis)

- GF-AFC EC$_{50}$ = 1.9nM
- bis-AAF-R110 EC$_{50}$ = 1.9nM
- Caspase-Glo EC$_{50}$ = 1.7nM

![Graphs showing cellular responses to different assays and concentrations](image)
Detecting off-target toxicity by multiplexing MultiTox-Fluor followed by HDAC-Glo

Cardiomyocytes (Primary)

- Viability EC$_{50}$ = ND
- Cytotoxicity EC$_{50}$ = ND
- HDAC Activity IC$_{50}$ = 2.3nM

U937 (Transformed Cancer Cells)

- Viability EC$_{50}$ = 566nM
- Cytotoxicity EC$_{50}$ = 505nM
- HDAC Activity IC$_{50}$ = 64nM

[24hr compound exposure]
**Intrinsic and extrinsic apoptotic pathways terminating with activation of Caspase-3 → Apoptosis**

- **Intrinsic Pathway**:
  - Stress, DNA Damage, Chemotherapy, UV
  - Bcl-2
  - Smac/DIABLO
  - Cytochrome C
  - Apaf-1, Apoptosome
  - Caspase-9, Caspase-7, Caspase-3

- **Extrinsic Pathway**:
  - Death Ligands, Death Receptors
  - Caspase-8
  - Apollon, CIAP1, CIAP2, ILP-2, Livin, NAIP, Survivin, XIAP
  - Caspase-3, Caspase-7, Apoptosis

**Caspase-3 executioner protease** is a “universal” marker of apoptotic cell death.
**Multiplex assay of signaling and executioner caspases**

Combining fluorogenic and luminogenic protease substrates together

1. Add Caspase-Glo® 8 Reagent + (Z-DEVD)$_2$-R110
2. Incubate
3. Record Luminescence
4. Record Fluorescence

Graph showing the measurement of Caspase-8 and Caspase-3/7 over hours of drug exposure.
Mitochondrial toxicity can be detected by using controlled culture conditions

• Cells cultured in medium with galactose instead of glucose to eliminate production of ATP from glycolysis* (ATP production comes from mitochondrial oxidative phosphorylation)

• Expose cells to treatment less than 4hr

• Measure membrane integrity and ATP using sequential multiplex protocol

• Decrease in ATP without change in cell viability suggests mitochondrial toxicity

Mitochondrial ToxGlo™ Assay
Multiplex membrane integrity and ATP content

Treat cells
30 min - 4hr (glucose free)

bis-AAF-R110 Substrate

Incubate 30 min

Record Fluorescence

ATP Assay Reagent

Incubate 10 min

Record Luminescence

Expected Assay Profiles

No toxicity at this exposure period

Primary Necrosis

MitoTox Without necrosis

MitoTox with necrosis
Oxidative stress marker assay

- Reduced glutathione (GSH) serves as an antioxidant in cells
- Low levels of GSH are associated with oxidative stress
- GSH can be measured with a luminescent assay using Glutathione S Transferase (GST) and luciferase
- Fluorescent cell viability assays can be sequentially multiplexed with the luminescent GSH assay
How the GSH-Glo™ Assay works

- Glutathione S Transferase transfers GSH to nitrotoluene group and converts pro-luciferin to luciferin in a GSH dependent reaction
- Luciferase reaction generates light proportional to the amount of GSH in cells
Multiplexing GSH levels with measuring viability

MultiTox-Fluor™ Reagent

Incubate 37°C

Record Fluorescence

Remove medium

GSH-Glo™ Buffer, 30min

GSH-Glo™ Reagents, 15min

Record Luminescence

Viability EC$_{50}$ = ND

Cytotoxicity EC$_{50}$ = ND

Glutathione EC$_{50}$ = 188nM

![Graph showing EC$_{50}$ values for viability, cytotoxicity, and glutathione levels.](image)
Proteasome activity as protein turnover marker

• The proteasome is responsible for most turnover of cytoplasmic proteins
• The proteasome is a therapeutic target for cancer
• Proteasome inhibitors (e.g. Velcade) selectively induce apoptosis in cancer cells
• Proteasome-Glo™ Assays provide adequate sensitivity to enable cell-based assays in HTS format
• Multiplexing proteasome and caspase assays can provide information on mechanism of toxicity leading to apoptosis
Multiplexing proteasome and apoptosis assays

Caspase-3 activity is absent after 1.5 hrs treatment with proteasome inhibitor; but increases after 4.5 hours incubation.
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Luciferase reporters of cell stress pathways leading to cytotoxicity
Stress response pathways leading to cytotoxicity

• Stress response pathways are toxin activated signal transduction events that modulate transcription factors to trigger expression of cytoprotective genes to enable the cell to attempt to restore homeostasis.*

• Triggering cell response pathways occurs at lower toxin doses or exposure times than what is needed to directly trigger necrosis or apoptosis.

• If stress cannot be overcome to re-establish homeostasis, the result is induction of apoptosis and removal of the cell.

Luciferase reporters of cell stress pathways leading to cytotoxicity

- Luciferase reporter assays are ideal to measure effects of compounds on expression of stress pathway genes.
- Reporter assays can help identify the cellular mechanisms used to adapt to a perturbation, gaining insight into the mechanism of toxicity caused by the compounds.
- Promega has developed luminescent reporter gene assays directed at the major pathways involved in cellular stress.
- Several vectors and stable cell lines are available from Promega as “Latest Research Materials”
### Stress and toxicity pathway vectors

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<tr>
<th>Pathway/Response</th>
<th>Transcription Factor</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant</td>
<td>Nrf2</td>
<td>pGL4/ARE</td>
</tr>
<tr>
<td>DNA damage</td>
<td>p53</td>
<td>pGL4/p53</td>
</tr>
<tr>
<td>ER stress</td>
<td>ATF6</td>
<td>pGL4/ERSE</td>
</tr>
<tr>
<td>ER Stress</td>
<td>ATF4</td>
<td>pGL4/ATF4</td>
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<tr>
<td>ER stress</td>
<td>Xbp1</td>
<td>pGL4/Xbp1</td>
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<tr>
<td>Heavy metal stress</td>
<td>MTF1</td>
<td>pGL4/MRE</td>
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<tr>
<td>Heat shock</td>
<td>HSF1</td>
<td>pGL4/HSE</td>
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<td>Hypoxia</td>
<td>Hif1α</td>
<td>pGL4/HRE</td>
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<tr>
<td>p38/JNK</td>
<td>AP1</td>
<td>pGL4/AP1</td>
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<tr>
<td>Xenobiotic stress</td>
<td>AhR</td>
<td>pGL4/XRE</td>
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<tr>
<td>Inflammation</td>
<td>NFκB</td>
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<tr>
<td>Osmotic stress</td>
<td>NFAT5</td>
<td>pGL4/NFAT5</td>
</tr>
</tbody>
</table>

All constructs with pGL4.27 backbone [luc2P/minP/HygR]
Examples of major stress response pathways

**Oxidative Stress Response:** Signaling pathway that leads to Nrf2 transcription factor binding to antioxidant response elements (ARE) that induce expression of genes to neutralize Reactive Oxygen Species (ROS) and to limit oxidative damage to cellular components.

**Heat Shock Response:** HSF-1 activates expression of Hsp70 & Hsp27 chaperones that bind to and facilitate refolding of denatured proteins.
Examples of major stress response pathways (cont.)

**p53-mediated DNA Damage Response:** A genotoxic response pathway mediated by the p53 transcription factor to arrest the cell cycle, regulate DNA repair, and if the damage is too great, mediate apoptosis.

**ER Stress Response:** ATF6 directs transcription of endoplasmic reticulum (ER) specific chaperones and slowing of general protein synthesis to enable ER to refold unfolded proteins.
Oxidative stress response / ARE-luc2P (Nrf2)

Data collected 18 hrs post compound addition (pREBFB25/HEK293)

<table>
<thead>
<tr>
<th></th>
<th>ARE</th>
<th>D,L-sulf</th>
<th>tBHQ</th>
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</thead>
<tbody>
<tr>
<td>S/B</td>
<td>116</td>
<td></td>
<td>152</td>
</tr>
<tr>
<td>EC50 (μM)</td>
<td>19.6</td>
<td></td>
<td>NA</td>
</tr>
</tbody>
</table>
DNA damage stress response / p53-luc2P (p53)

Data collection 18 hrs post-treatment for all except mitomycin c (40 hrs)
ER stress response / ERSE-luc2P (ATF6)

Data collected 23 hrs post addition of tunicamycin (pREBFB28/HeLa)
Several stress response reporters can be tested in parallel

Protocol to profile a compound against RE panel

- Add each DNA to FuGENE® HD in Opti-MEM I®
- Add cells to transfection complexes
- Dispense cells into 96-well plate, with each RE in its own column
- Incubate overnight
- Treat cells 18 hr with varying doses of the compound of interest
- Multiplex assays for cell viability and reporter induction
Multiplex cell viability & cell stress reporter genes

CellTiter-Fluor™ Reagent (GF-AFC)

Incubate 37°C 15-30 min

Record Fluorescence

ONE-Glo™ Reagent

Record Luminescence

HepG2 cells treated 18 hrs
Tunicamycin selectively stimulates ATP6 Response Element

Fold Response

log [tunicamycin] (M)
Excellent multiplexing partners for live cells or dead cells

**CellTiter-Fluor™ Assay**
- Reaction occurs within living cells.
- Live cell protease is inactive outside cells.
- AFC fluorescence is proportional to live cells

**CytoTox-Fluor™ Assay**
- Rhodamine 110 substrate cannot enter cells.
- Reaction occurs in the culture medium.
- Rhodamine110 fluorescence is proportional to dead cells.

Read about the development of these assays:
Multiplexing luminescent & fluorescent assays

CellTiter-Glo® Luminescent Cell Viability Assay

CytoTox-Fluor™ Cytotoxicity Assay

CellTiter-Fluor™ Cell Viability Assay

MultiTox-Fluor

ONE-Glo™ + Tox Assay

ApoTox-Glo™ (3/7)

ApoLive-Glo™ (3/7)

Caspase-Glo® Assays

MultiTox-Glo

CytoTox-Glo™ Cytotoxicity Assay

GSH-Glo™ & GSH/GSSG-Glo™ Assays

P450-Glo™ Cell-Based Assays

Proteasome-Glo™ Cell-Based Assays

All possible with the GloMax®-Multi & -Multi+ Detection Systems
Summary

• There is a variety of multi-well plate assay options available to detect more than just whether cells are alive or dead.

• Biochemical and cell-based assays are available to detect cell stress events (e.g. oxidative stress, mitochondrial toxicity, proteasome, etc.).

• Luciferase reporter gene assays and stable cell lines have been developed to study effects on the major stress pathways leading to cytotoxicity.

• Multiplex detection of cell viability in combination with luciferase reporters or other biochemical marker assays provides powerful tools to study the mechanisms leading up to cytotoxicity.
## Product names for assays described today

<table>
<thead>
<tr>
<th>Assay Technology</th>
<th>Promega Product Name</th>
</tr>
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<tbody>
<tr>
<td>MTT</td>
<td>CellTiter 96® Non-Radioactive Cell Proliferation Assay</td>
</tr>
<tr>
<td>MTS</td>
<td>CellTiter 96® AQ&lt;sub&gt;ueous&lt;/sub&gt; One Solution Cell Proliferation Assay</td>
</tr>
<tr>
<td>Resazurin</td>
<td>CellTiter-Blue® Cell Viability Assay</td>
</tr>
<tr>
<td>Protease marker</td>
<td>CellTiter-Fluor™ Cell Viability Assay</td>
</tr>
<tr>
<td>ATP</td>
<td>CellTiter-Glo® Luminescent Cell Viability Assay</td>
</tr>
<tr>
<td>LDH</td>
<td>CytoTox-ONE™ Homogeneous Membrane Integrity Assay</td>
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<tr>
<td>Protease release</td>
<td>CytoTox-Fluor™ Cytotoxicity Assay</td>
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<tr>
<td>DNA staining</td>
<td>CellTox-Green (Inquire; under development)</td>
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<td>Caspase-3/7 activity</td>
<td>Caspase-Glo® 3/7 Assay</td>
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<tr>
<td>Caspase-8 activity</td>
<td>Caspase-Glo® 8 Assay</td>
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<td>Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay</td>
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<tr>
<td>Live+Dead+Apoptotic</td>
<td>ApoTox-Glo™ Triplex Assay</td>
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<tr>
<td>Stress response pathways</td>
<td>Luciferase reporters of cell stress pathways (Inquire in Latest Research Materials section of promega.com)</td>
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Questions Welcome